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Evaluation of multidrug efflux pump expression in clinical isolates of *Staphylococcus.aureus*

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ABSTRACT

Background: One of the mechanisms of *Staphylococcus aureus* resistance to ciprofloxacin is the presence of efflux pumps. The aim of this study was to determine the frequency and expression of *norA*, *norB* and *norC* efflux pump genes in ciprofloxacin-resistant *Staphylococcus aureus* clinical isolates.

Materials and methods: In this study, 40 isolates of *S. aureus* were obtained from blood and urine samples. Antibiotic resistance pattern by disc diffusion method and the minimum inhibitory concentration (MIC) was evaluated. Identification and expression of nor efflux family genes (*norA*, *norB*, and *norC*) in ciprofloxacin-resistant isolates of *S. aureus* were determined using Real-Time PCR method.

Results: The resistant to ciprofloxacin, Gentamicin, Cotrimoxazol, Erythromycin and Amoxicillin was 42.5%, 12.5%, 12.5%, 50% and 87.5%, respectively. All of ciprofloxacin-resistant isolates were positive for of *norA*, *norB*, and *norC* genes. The results of Real-Time PCR showed that all ciprofloxacin-resistant isolates are active in the case of efflux pumps. Isolates showed different expressions of the *norA*, *norB* and *norC* genes. Expression levels of *norA* and *norB* increased by 6.8 and 7.1 fold, respectively.

Conclusion: The results indicated that nor efflux family genes were expressed in all ciprofloxacin-resistant isolates of *S. aureus*. Moreover, there is an association between the expression of the efflux pump genes and resistance to ciprofloxacin in *S. aureus* isolates.

1. Introduction

Staphylococcus aureus is a bacterial pathogen from the Micrococcaceae family, which is a common bacterial pathogen in hospital-acquired infections around the world. The anterior portion of the nose is the primary source of *S. aureus* in adults and children, and between 20% to 40% of healthy individuals are the carriers of this bacterium (Corredor Arias et al., 2016).This bacterium causes a wide range of diseases including pneumonia, skin infections, endocarditis, and osteomyelitis (Hefzy et al., 2016; Osman et al., 2016). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the opportunistic pathogens in the hospital whose resistance to methicillin is mediated by producing a specific penicillin-binding protein called PBP2a encoded by the *mecA* gene (Petrović-Jeremić et al., 2016; Udobi et al., 2013). Today, MRSA strains are resistant to most conventional β-lactam antibiotics, such as oxacillin, which limits the treatment of MRSA-mediated diseases (Gómez et al., 2016; Ersoy et al., 2019). Fluoroquinolone drugs such as ciprofloxacin are one of the most appropriate and alternative drugs for the treatment of MRSA strains (Firsov et al., 2008).

However, following administration of this antibiotic for the treatment of infections, bacterial resistance to antibiotics has occurred. In some cases, the resistance level was 100% (Mustapha et al., 2016).

Antibiotic resistance mechanisms are different in *S. aureus* strains. One of the mechanisms is to prevent drug accumulation inside the cell through efflux pump systems which transport toxic substances such as antibiotics into the environment (Poole, 2007; Li and Nikaido, 2009; Kosmidis et al., 2012; Paulsen and Lewis, 2002).

Antimicrobial efflux systems have been classified into five families based on their energy source and structure namely, the small multidrug

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resistance (SMR) family; the major facilitator superfamily (MFS); the resistance-nodulation-cell division (RND) superfamily; the multidrug and toxic compound extrusion (MATE) family; and the adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily (Li and Nikaido, 2009; Soto, 2013; Costa et al., 2013). Efflux pumps are effectively relevant to the major facilitator superfamily (MFS) and the resistance-nodulation-cell division (RND) superfamily that contribute to the removal of antibiotics using the proton motive force (Li and Nikaido, 2009).

The MFS transport system is one of the main efflux systems in *S. aureus* and NorA pump is one of the major pumps in this family. The efflux pump NorA is encoded by the chromosomal gene *norA* and contains 388 amino acids. Several studies have shown that norA can transport various compounds such as hydrophilic fluoroquinolones including norfloxacin, ciprofloxacin, ethidium bromide, and quaternary ammonium compounds outside the cell (Yoshida et al., 1990; Noguchi et al., 2004). Researchers have shown that the *nor*A gene has a basal expression in cells causing a low resistance to antibiotic compounds. Increased resistance to fluoroquinolones is associated with an overexpression of norA efflux pump (Hooper and Jacoby, 2015).

The norB pump is another MFS proton-driven efflux pump in *S. aureus*, which is encoded by the chromosomal gene *norB* and contains 463 amino acids. It is responsible for the resistance to fluoroquinolones and biocides. Previous studies have shown that this pump is also important in increasing the pathogenicity of this bacterium (Truong-Bolduc et al., 2005).

The norC pump belongs to the MFS family and is encoded by chromosomal gene *norC* and contains462 amino acids. NorC contributes to low-level resistance to hydrophilic and hydrophobic fluoroquinolones such as garenoxacin, moxifloxacin, and ciprofloxacin (Li and Nikaido, 2009; Hooper and Jacoby, 2015).

In the clinical approaches, the first choice for the treatment of MRSA infections is the ciprofloxacin antibiotic. Today, the biggest challenge and concern in hospitals is a hospital-acquired infection (HAI) with opportunistic *S. aureus*, which has been resistant to both methicillin and ciprofloxacin antibiotics.

The purpose of the present study was to evaluate the expression levels of efflux pump genes including *norA*, *norB*, and *norC* in ciprofloxacin-resistant isolates.

2. Material and methods

2.1. Bacterial strain identification

In this descriptive study, a total of 40 *S. aureus* isolates were recovered from blood and urine samples from Imam Khomeini Hospital, Tehran, Iran during the second 6 months of the year 2018. Isolates were identified using Gram staining, catalase test, Mannitol fermentation and DNase and detection of the *grlA* gene by PCR.

2.2. Antimicrobial susceptibility testing

After identifying *S. aureus* isolates, the antibiotic susceptibility of bacteria was investigated by a disc diffusion method against ciprofloxacin (5 μg), Gentamicin (10 μg), Cotrimoxazol (25 μg), Erythromycin (15 μg), and Amoxicillin (30 μg) (UK, MAST) in Hinton Muller agar medium (Merck, Germany). The results were interpreted according to CLSI (Clinical and Laboratory Standards Standard 2015, Institute) guidelines.

The susceptibility of strains to ciprofloxacin was rechecked by broth micro dilution method in triplicates in accordance with CLSI guideline.

2.3. Primer design

The sequences of primers in this study were designed by Oligo 7 and Primer 3 software (Table 1) (Rychlik, 2007).

2.4. PCR amplification

DNA extraction was done using Genomic DNA Extraction Kit (Bioneer, Republic of Korea). The presence of *norA*, *B*, and *C* genes in isolates were tested by PCR, using the primers described in Table 1. The PCR reaction mixture with the final volume of 20 μl was prepared and DNA amplification was performed in a thermal cycler with denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 20 s plus a final extension at 72 °C for 4 min.

2.5. Analysis of norA, norB and norC gene expression levels by Real-Time PCR

The sub-MIC concentration of ciprofloxacin was used for RNA extraction. The bacteria in the sub-MIC were collected and pelleted through centrifugation at 2500 $\times g$ for 15 min. RNAs were extracted via the RNeasy Plus Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

Subsequently, cDNA synthesis was performed using Easy cDNA reverse transcription (Parstous, Iran). Evaluation of *norA*, *norB*, and *norC* expression levels was performed by quantitative time-real PCR (qPCR), in Rotor-Gene thermal cycler (Corbett 6000; Australia). A final volume of 20 μl PCR reaction mix containing 2 μl of cDNA, 10 μl SYBR Green master mix, 6 μl nuclease-free water and 1 μl of each primer (Table 1) was run according to the following program: initial activation step at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 20 s. The 16srRNA gene was used as an internal control to normalize target genes expression measurements. Real-Time PCR results were analyzed using the 2(−Delta Delta C(T)) method (Livak and Schmittgen, 2001).

2.6. Data analysis

Data were analyzed with Mann Whitney *U* test using GraphPad Prism 6 program. The *P-value* for statistical significance is defined as $P < 0.05$.

3. Results

In the present study, a total of 40 *S. aureus* isolates were confirmed by different biochemical tests and PCR using specific primer for *grl*A gene. Twenty-nine (72.5%) of *S. aureus* isolates were obtained from 29 blood samples and 11 isolates were obtained from urine sample.

The susceptibility test results of *S. aureus* in the study showed 42.5% resistance to ciprofloxacin, 12.5% resistance to Gentamicin, 12.5% resistance to Cotrimoxazol, 50% resistance to Erythromycin, and 87.5% resistance to Amoxicillin.

In the next step, the minimum inhibitory concentration of ciprofloxacin was performed by microdilution broth for 40 isolates. According to MIC results, 17 isolates (42.5%) were resistant to ciprofloxacin. The MIC results showed that out of 17 fluoroquinolones resistant isolates using disc diffusion method, 6 isolates (35%-6/17) had the minimum inhibitory concentration in the resistant range (MIC ≥ 1 mg/ml), 3 isolates (17%-3/17) with the minimum inhibitory concentration in the semi-sensitive range (MIC = 0.12–0.5 mg/ml) and 8 isolates (47%-8/17) had the minimum inhibitory concentration in the sensitive area (MIC ≤ 0.06 mg/ml) (Table 2).

3.1. PCR and Real-Time PCR

In order to investigate the presence of efflux pump genes in isolates from *S. aureus*, specific primers for these genes were designed. The expected PCR bands size for *norA*, *norB*, and *norC* genes were 121 bp, 160 bp, and 132 bp, respectively which were observed in all 15 resistant isolates. In Real-Time PCR assay, all isolates in sub-MIC concentration

Table 1
Primer used in qPCR.

Genes		Primer sequence (5′ → 3′)	Amplicon (bp)	Tm (°C)	References
norA	F	AATGCCTGGTGTGACAGGTT	121	60	This study
	R	TCCACCAATCCCTGGTCCTA			
norB	F	AAAAGCCGTCAAGAGAGGCA	160	60	This study
	R	ACGGCGATATTAACCGTTCCA			
norC	F	GTTCTTGGGGTGGAAGTGGT	132	60	This study
	R	CAGGCGTCCCTTTGATGAGT			
16srRNA	F	ATGGTTGTTGCAAACCTGCC	117	60	This study
	R	GCGTTCGCCCACTTCTTTTT			

Table 2
Minimum inhibitory concentration of ciprofloxacin among isolates.

Break point	Ciprofloxacin		
	Resistance (≥1)	Intermediate (0.12–0.5)	Sensitive (≤0.06)
MIC (μg/ml)	≥1	0.12–0.5	≤0.06
Isolates	6	3	8
Total	17		

showed a different expression of efflux pump genes. There was a statistically significant difference between the expression of *norA* and *norB* genes in comparison with expression of *16s*rRNA gene as internal control ($p \leq 0.05$).

In isolates with sub-MIC concentration, the expression of *norA* and *norB* genes were similarly increased by 6.8 and 7.1 fold, while the expression of *norC* gene was low and 2.8 fold increased in comparison to the control (Fig. 1). Real-Time PCR data showed that in resistant isolates, norA and norB pumps may play a more important role than norC in resistance to ciprofloxacin.

4. Discussion

The mechanism of resistance to quinolones is different in bacteria. In *E. coli* bacterium, the mechanism of resistance to quinolones is due to the alteration of the DNA structure of the gyrase (Nakanishi et al., 1998). As mentioned earlier, one of the mechanisms of resistance to ciprofloxacin in strains of *S. aureus* is the presence of efflux pumps (Hassanzadeh et al., 2017). These pumps transport a wide range of materials including antibiotics, antiseptic compounds, dyes, and detergents. Therefore, they play a significant role in the development of multi-drug resistance. In general, this efflux pump genes are located on chromosome and are conserved among the strains.

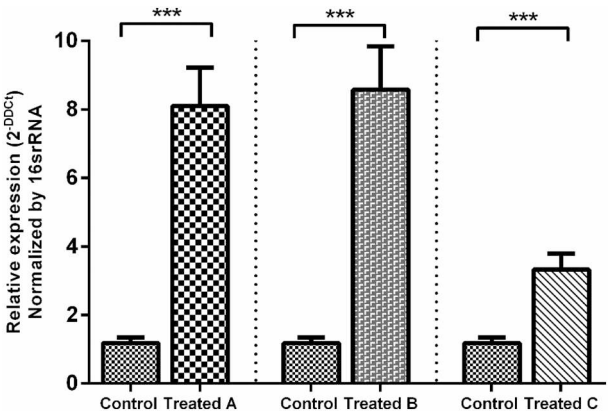

Fig. 1. Relative expression of *norA*, *B* and *C* efflux pump genes in clinical isolates of *S.aureus*. In ciprofloxacin-resistant isolates, the expression of *norA*, *norB* and *norC* genes increase > 6.8, 7.1 and 2.8 fold, respectively. Asterisk indicates significant difference between two groups by Mann Whitney *U* test ($P < 0.05$).

In recent years, resistance to quinolones has been reported in MRSA strains (Moreno-Flores et al., 2018). In the United States, ciprofloxacin-resistant MRSA strains developed three months after using ciprofloxacin (Motallebi et al., 2016).

As noted, MRSA strains are one of the most important pathogens in hospitals that spread rapidly worldwide. This organism is resistant to most beta-lactam antibiotics and there are few substitutes for this drug (Ghosh and Banerjee, 2016). One of the alternative therapies is the ciprofloxacin therapy from the quinolone family; however, recent studies have shown increased resistance to this antibiotic. In this study, among 40 clinical isolates, 17 isolates were ciprofloxacin-resistant (42.5%) by micro dilution method. The difference between the result of disc diffusion and broth micro dilution methods shows that MIC detection methods can be more accurate and more sensitive than disc diffusion methods.

Different studies have been conducted on the phenotypic and genotypic identification of efflux pumps in *S. aureus* strains. Present study was performed to evaluate the frequency of *norA*, *norB*, and *norC* efflux pump genes and their expression in ciprofloxacin-resistant isolates of *S. aureus*.

In our study, *norA*, *norB* and *norC* genes were present in all ciprofloxacin resistant isolates in MIC method. Similar results are reported in other studies. Hoove et al. (Huet et al., 2008) tested 9 ciprofloxacin resistant MRSA strains for the presence of efflux pumps and expression in the presence of low concentrations of antibiotics using Real-Time PCR method. The results of this study showed that *nor*A and *nor*B genes were present in all resistant strains and their expression increased along with the increase in antibiotic concentrations.

Pourmand et al. (2014) also checked the presence of *nor*A pump gene and its expression in ciprofloxacin-resistant strains by Real-Time PCR method. The results of this study showed that *nor*A gene exists in all ciprofloxacin-resistant isolates and its expression is increased in the presence of the biocide hexahydroquinolone.

Our results are in agreement with previous studies, resistance to ciprofloxacin have been increased among the clinical isolates of *S. aureus*. *nor*A, B, and C were present in all resistant isolates and their expression increased in comparison to the control.

The results of this study showed that norA and norB is one of the most important mechanisms in resistance to fluoroquinolone antibiotics such as ciprofloxacin, but the role of other factors and mechanisms involved in resistance should not be ignored. Finally, further studies are essential for production and the expansion of new anti-oxidant-inhibiting molecules. The development of efflux pumps inhibitors makes it possible to control resistant strains.

Declaration of competing interest

No conflict of interest is declared.

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Ethical permissions

The Ethics Committee of Islamic Azad University, Tehran, Iran confirmed the present research.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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